Immunologic Enhancement of Allogeneic Tumor Growth with Soluble Histocompatibility-2 Antigens
(tumor-specific transplantation antigen/mice/solubilization/immune tolerance)

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ABSTRACT Soluble, partially purified, histocompatibility antigens that were obtained from the membranes of A/J spleen cells have been assayed for their capacity to elicit immunologic enhancement of two tumors of A-strain origin: YAA-C1 and Sarcoma I. Crude membrane material and a partially purified, soluble antigen that were contained in a specific fraction, obtained after chromatography on a Sephadex G-150 column, elicited enhancement; this fraction has been shown to contain immunogenic histocompatibility-2a antigens as well as alloantigenic specificities that were detected serologically. Another soluble fraction did not induce enhancement; this fraction has been shown to contain antigens other than H-2.

Passive enhancement of both tumors was achieved with antisera produced in allogeneic mice that were inoculated with crude membrane material or with a fraction obtained by Sephadex G-150 chromatography. These antisera contain cytotoxic and/or hemagglutinating antibodies. Immunologic enhancement was specific. A readily enhancementable tumor, Py 89, of C57BL origin was not enhanced with anti-H-2a antisera.

These results suggest strongly that all important H-2a transplantation antigenic determinants of spleen cells can be recovered by partial papain digestion and fractionation on a Sephadex G-150 column.

Mouse histocompatibility antigens are membrane-bound; they elicit transplantation immunity and are controlled by multiple genetic loci. The major histocompatibility locus of the mouse, H-2, has more than 20 known alleles; each allele determines a different combination of antigenic specificities (1). Although solubilization and extensive purification of histocompatibility antigens of certain H-2 genotypes have been achieved, antigenic activity has been monitored, to the present, principally by serologic methods (2-4). A major concern, therefore, is whether antigens detected serologically are able to elicit specific immunity with consequent graft rejection.

We have shown (5) that fractionation of papain-solubilized cell membranes obtained from strain A/J (H-2^a) spleen cells, using Sephadex G-150, yielded three fractions, monitored by 280-nm absorbance; fraction 1, in the excluded volume and fractions 2 and 3 in the included volume. All fractions were tested for alloantigenic activity by an in vivo assay: inhibition of immune cytolysis of lymph node cells labeled with ^51Cr; in addition, the three fractions were assayed for the presence of transplantation antigens by an examination of their ability to elicit transplantation immunity both in noncongeneric strains of mice that differ at H-2 and other loci and in congenic strains of mice, which differ only at the H-2 locus. It was found that all of the H-2a serologic specificities assayed were confined to a single peak (fraction 2); only this fraction accelerated skin-graft rejection in the congenic strains of mice that differ at the H-2 locus. The activity of the F2 fraction was found to be immunologically specific. The association of H-2 alloantigenic specificities (tested in vivo) and transplantation antigens (tested in vitro) with fraction F2, strongly suggests that both the serological specificities and the transplantation antigens are located either on the same molecules or on molecules of similar molecular weight. Our studies did not provide critical evidence for the completeness of the antigen. The techniques used may detect haptons as well as complete antigens.

In this study, we have assayed the biologic capacities of our solubilized-membrane materials to induce immunologic enhancement, that is the prolonged growth and survival of tumor allografts due to the presence in the graft recipient of alloantibodies directed against the alloantigens of the graft donor. Present evidence suggests strongly that in order to achieve enhancement, the appropriate alloantibodies must combine with all the determinants of the transplantation antigens (6, 7). Specific immunologic enhancement is therefore a test for completeness of the antigen. This report describes the effects of the F2 and F3 fractions as well as crude membrane preparations from A/J (H-2^a) spleen cells on the growth and regression of two A/J strain tumors: YAA-C1 grafted in CBA (H-2^b) mice and Sarcoma I grafted in mice of the B10.D2 (H-2^b) strain.

MATERIALS AND METHODS

Antigenic Materials. Preparation of the crude membrane material and treatment with papain were performed as described (5). Batches of 1000 spleens from A/J mice have been routinely used as a starting material and the papain-solubilized material, after concentration by ultrafiltration, was chromatographed on a Sephadex G-150 column (2.5 x 160 cm), maintained at 4°C. H-2 alloantigenic specificities measured as described (5) by a modification of the in vitro (^51Cr release) cytotoxic inhibition assay were confined to a middle peak (fraction 2). Material pooled from this fraction with a specific activity comparable to that reported for H-2.1,3 and H-2.11 was used throughout this study. Antisera directed against H-2 specificities as used in the cytotoxic inhibition assay were obtained from the Jackson Laboratory through NIAID, Bethesda, Md. Protein concentrations were

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determined by the method of Lowry et al. (8) with crystalline
plasma albumin as a standard.

Mice. A/J (H-2b) and B10.D2 (H-2d) strains of mice were
purchased from the Jackson Laboratory, Bar Harbor, Me.
CBA (H-2k) mice were taken from stocks maintained in our
laboratory. The original nucleus of this stock was obtained
from Dr. A. C. Allison, the National Institute for Medical
Research, London, and has been maintained by strict broth-
sister matings. Additional strains of mice used in the antigenic
specificity studies were BALB/c and C3Hf/Lw; both strains
were raised in our own colony. These mice were used in the
production of antisera.

Antibody Titrations. Sera of CBA and B10.D2 mice that
were injected with antigenic material were assayed for cyto-
toxic and hemagglutinating antibodies at the times indicated
in the tables and text.

Diluted sera were incubated for 30 min at room tempera-
ture, with 5 × 10^3 A/J lymph node target cells that were
labeled with ^1^HCr. Absorbed guinea pig complement was then
added and incubation was continued for an additional 90 min.
Release of ^1^HCr was measured by an automatic gamma-cell
counter (Nuclear-Chicago). Specific cytotoxic titers are ex-
pressed as the reciprocal of the dilution of antiserum that lysed
50% of lymph node target cells. An antiserum directed against
H-2^d^ specificity H-2.23 gave a cytotoxicity reciprocal titer of
96.

Hemagglutinins of the four groups of sera obtained from
B10.D2 mice were assayed by the method of Stimpfling et al.
(9).

Tumors. The ascitic form of Sarcoma I was obtained origi-
nally from Dr. N. Kaliss, Jackson Laboratory and was carried
by intraperitoneal passage in A/J strain mice. The enhance-
ability of this tumor has been reported in several studies (10-
12). The ascitic form of tumor YAA-C1 was obtained from
Dr. K. E. Hellström (13). It is a reticulum-cell sarcoma of an
A-strain origin that was induced by Moloney leukemogenic
virus and acquired a polyoma-specific (tumor-specific trans-
plantation, TST) antigen after repeated infections with
polyoma virus. Tumor Py 89 is a fibrosarcoma induced in
C57Bl/KaLw mice by polyoma virus and has been adapted to
in vitro growth (14). All three tumors grew progressively
and killed their syngeneic recipients.

Preparation and Incubation of Tumor Cells. YAA-C1 and
Sarcoma I ascites cells were washed twice in medium 199.
10^6 cells of tumor YAA-C1 or 6.2 × 10^6 cells of Sarcoma I
in 0.2 ml of medium 199 were inoculated subcutaneously into
each mouse. The Py 89 cells that were propagated in vitro,
were trypsized and inoculated subcutaneously at a concen-
tration of 5 × 10^6 cells per mouse in 0.1 ml of medium 199.

RESULTS

Active and passive enhancement of YAA-C1 tumors

Equal numbers of male and female CBA mice, 8–11 weeks of
age, were inoculated intraperitoneally with the following
materials: (a) crude membrane preparation at concentrations
of 50 and 100 μg per 0.2 ml, of medium 199 emulsified with
equal portions of Freund’s complete adjuvant, were injected
into males and females, respectively; (b) Fractions 2 or 3 at
concentrations of 50 and 100 μg, emulsified with Freund’s
adjuvant and injected as in (a); (c) control mice were injected
with 0.2 ml of 0.01 M Tris·HCl buffer (pH 8.4)–0.15 M NaCl–
Freund’s adjuvant. Booster inoculations were given 2 weeks
later; the same concentrations and volumes of inoculum were
used without Freund’s adjuvant. All mice were then chal-
enged subcutaneously, 10 days after the booster inoculations,
with YAA-C1 ascites cells.

YAA-C1 tumor cells grew in all CBA hosts to a size of
about 10 × 10 × 20 mm, by the eighth day after grafting.
Subsequently, either progressive growth and death of mice or
complete regression was observed; in many individuals, how-
ever, a delayed regression occurred, even after the tumors
attained substantially large sizes. Table 1 shows that both crude
membrane material and fraction 2 induced the capacity of
nearly 50% of the CBA recipients to support the growth of
YAA-C1 cells, which caused death of the host from the tumor.
Equal numbers of female and male mice were used; the results
were combined in Table 1. In two groups, however, not shown
separately in the table, a higher susceptibility of females to
progressive growth and a delayed regression of the tumor was
observed. 25 days after challenge with the tumor, all six non-
immunized (adjuvant inoculated) CBA female mice com-
pletely rejected their tumors; eight female mice that were
treated with fraction 2 showed progressively growing neo-
plasms. This contrasts to the usually greater susceptibility of
male recipients to enhancement and correlates with their
usually less vigorous immune response to transplantation
antigens. We did not observe however, in our previous study,
a lowered immune responsiveness among CBA males, which
were injected with fraction 2, to allogeneic (Strain A) skin
grafts. In the present work, the higher concentrations of cell-
membrane preparations and fraction 2 (100 μg) were given to
females, which may account for their heightened suscepti-
ability to YAA-C1 cells.

CBA recipients, treated with 50 or 100 μg of fraction 3, did
not differ from untreated controls, which were injected with
Freund’s adjuvant alone. Fig. 1A shows comparative rates of
regression over a 75 day period.

That active immunologic enhancement may account for the
increased frequency of progressive tumor growth and the
delayed regressions in the two groups of CBA recipients of
cell-membrane and fraction-2 preparations (both contain
H-2 isoantigens) is suggested by the results shown in Table 2.
Specific serum antibodies are known to mediate immunologic
enhancement and H-2 isoantigens play a major role. Pooled
antisera from four CBA mice injected with fraction 2 and
showing enhanced tumor growth gave strikingly increased
cytotoxic titers over CBA control mice that were injected

<table>
<thead>
<tr>
<th>Table 1. Fate of YAA-C1 tumor cells in CBA hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Crude membrane</td>
</tr>
<tr>
<td>Fraction 2</td>
</tr>
<tr>
<td>Fraction 3</td>
</tr>
<tr>
<td>Adjuvant controls</td>
</tr>
</tbody>
</table>

* Results at 75 days after inoculation.
† P < 0.02 for mice injected with fraction 2 compared to mice injected with fraction 3 or adjuvant.
peritoneally into six CBA mice 12 weeks of age, 30 min before grafting of 1 × 10^6 YAA-C1 tumor cells. Sera obtained from CBA recipients of the Freund's adjuvant were used as a control since enhanced growth was not observed in this group (group 4, Table 1). The cytotoxic titer of the serum from recipients of fraction 2 was 1/128 and that of the control serum was 1/8. The frequency of regression of the YAA tumor with time is shown in Fig. 1B. Regression of the YAA-C1 tumor was different in the two groups. At 25 days after grafting, for example, when 50% of the control mice had rejected their tumors, all of the recipients of alloantiseria from mice inoculated with fraction 2 bore tumors; one died with a progressively growing tumor. It is clear that passive transfer of antiserum from CBA mice that were injected with fraction 2, provided for enhanced growth of this allogeneic tumor.

**Active and passive enhancement of Sarcoma I**

B10.D2 (H-2^b^) strain mice, 16 weeks of age, were inoculated intraperitoneally with crude membrane (Group I), fraction 2 (Group II), fraction 3 (Group III), or Tris-buffered saline (Group IV); six males in each group received 50 μg, twice a week for 3 weeks of each material in buffered saline for a total of 300 μg and 6 females in each group received 100 μg of each material, twice a week for 3 weeks. Pooled sera from each group were obtained by retro-orbital sinus bleedings on days 13 and 27 after the last injection of material. Sarcoma I ascitic cells were inoculated 28 days after the last injection with the antigenic materials. Mice were observed daily for sarcomatous growths.

The results in Fig. 2A show that only those B10.D2 mice that were preexposed to crude membrane material or to the soluble fraction 2 were capable of sustained growth of Sarcoma I cells for a period of time. Results from male and female mice are combined since no essential differences were observed. Tumors in four of 10 mice injected with the fraction 2 attained volumes of about 10 × 10^2 mm at 17 days before finally regressing. One recipient died with a progressively growing tumor, 30 days after grafting. The response of B10.D2 mice, which were inoculated with crude membrane material differed from that of the mice that were injected with fraction 2.

An early growth of Sarcoma I was observed at 16 days. This tumor reappeared after regressing, along with a second graft, 47 days after the initial challenge with Sarcoma I ascitic cells. Both tumors grew progressively and killed their recipients at 90 days. Sarcoma I did not "take" in any of the B10.D2 recipients of fraction 3 nor in mice that were injected only with Tris-buffered saline.

The serum pools obtained on days 13 and 27 after inoculation with fraction 2 were individually assayed against erythrocytes and lymph node cells from strain A of mice, respectively for hemagglutinin and cytotoxic activities (Table 3). Only those groups capable of accepting the tumor graft and

**Table 2. Cytotoxic antibody in sera of CBA mice**

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Reciprocal of cytotoxic titers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at days:</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>128</td>
</tr>
<tr>
<td>Adjuvant controls</td>
<td>16</td>
</tr>
</tbody>
</table>

* Mice were inoculated with fraction 2 (see Methods).
sustaining progressive growth of the tumor for a period of time (groups 1 and 2) had significant titers of hemagglutinating and cytotoxic antibodies.

**Specificity of fraction 2-induced enhancement**

That enhanced growth of Sarcoma I and YAA-C1 tumors, provided by our soluble H-2* alloantigen preparation is specific, is shown by the results in Table 4. Fibrosarcoma Py 89 of C57BL origin was easily enhanced in allogeneic B10.D2 and CBA mice by passive transfer of Anti-C57BL or anti-Py 89 antisera that were produced in appropriated strains (groups 2 and 6), yet antisera produced in B10.D2 and CBA mice against the H-2* alloantigens of the soluble fraction 2 did not provide enhancement (groups 1 and 4).

**DISCUSSION**

We have solubilized H-2 antigens of A/J (H-2*) spleen cells by limited papain digestion. The partially purified fraction 2 eluted from a Sephadex G-150 column has been shown to be complete with respect to all H-2 serologic specificities tested [1,3,4,5,11,23, and 28] and, in addition, to be immunogenic as measured by a sensitive skin graft-rejection assay; H-2 activity was confined to fraction 2, found in the included volume (5). The association of alloantigenic specificities and transplantation antigens that are controlled by H-2 locus, suggests that both are present on the same molecule. Our previous studies did not provide evidence for the completeness of the solubilized H-2 transplantation antigens.

One test for the completeness of these antigens is the induction of immunologic enhancement. Enhancement may be either active, induced by immunization of the graft recipient with donor tissue or passive, by transfer to the recipient of serum produced by immunization of another individual with donor antigens. A strict temporal pattern is necessary to realize optimal enhancement; challenge with the tumor should occur 2-4 weeks after sensitization (11), at a time when cytotoxic or hemagglutinating antibodies reach peak titers. The enhancing potency of antisera appears to be positively correlated with these antibody titers (10). The enhancement, detected most effectively with allogeneic tumor grafting, results from the presence of alloantibody directed, principally against the H-2 transplantation antigens (11, 15) of the graft donor. Previous studies have demonstrated that effective enhancement can only be achieved when all H-2 antigenic sites on a tumor allograft or normal tissue, are covered with corresponding antibodies, thus enhancement is shown to be immunologically specific (6, 7).

The present studies were designed to test the ability of solubilized H-2 transplantation antigens, derived from strain A/J (H-2*) to enhance the growth of two tumor allografts: YAA-C1 (H-2*) and Sarcoma I (H-2*) in CBA (H-2*) and B10.D2 (H-2*) recipients, respectively. The experimental scheme was similar to that described initially by Kaliss (11).

The experiments show that specific immunological enhancement has been achieved on the basis of the following criteria:

(a) Active enhancement was noted in both systems studied with crude membrane material or fraction 2 but not with the fraction 3; both crude membrane preparation and fraction 2 are known from (5) to contain H-2 antigens. The soluble fraction 3 that was assayed previously was shown to be immunogenic in causing strain A (H-2*) skin-graft rejection in CBA (H-2*) recipients, but was not immunogenic when congenic recipients, differing only at the H-2 locus, were used (1). This observation suggested that the transplantation antigens in this fraction were controlled by loci other than H-2. The absence of enhancing capacity in fraction 3 further confirms this conclusion and reemphasizes the major role of H-2 antigens in immunologic enhancement. Cytotoxic and/or hemagglutinating antibodies were detected in the sera of those recipients of the cell-membrane preparation or fraction 2.

**Table 3. Hemagglutinating antibodies in B10.D2 mice**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Log₂ titers* at day:</th>
<th>13</th>
<th>27</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM fraction</td>
<td>256*</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>Fraction 2</td>
<td>256</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>Fraction 3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline controls</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Reciprocals of hemagglutinin titers. Reciprocal cytotoxicity titers of the several groups on day 27 were as follows for groups 1 through 4, respectively, 32, 32, 2, and 2. The reciprocal of cytotoxicity titers continued to increase after inoculation of 1 × 10⁶ Sarcoma I cells, as follows: day 37, group 2 (tumor-free) = 128, group 2 (with tumors) = >256, group 4 = 64; day 45, group 2 (tumor-free) = 80, group 2 (with tumors) = >1280, group 4 = 20.

**Table 4. Failure of anti-A (H-2*) antisera to enhance the growth of fibrosarcoma Py 89 (H-2*) of C57BL origin**

<table>
<thead>
<tr>
<th>Recipient mice of Py 89 cells and antisera</th>
<th>Group</th>
<th>Antisera*</th>
<th>No. of palpable tumors on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.D2 (H-2*)†</td>
<td>1</td>
<td>B10.D2 anti-A (fraction 2)</td>
<td>4/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BALB/c anti-C57BL</td>
<td>5/5 5/5 5/5 4/5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>C3H anti-Py 89</td>
<td>3/5 1/5 0/5 0/5</td>
</tr>
<tr>
<td>CBA (H-2*)</td>
<td>4</td>
<td>CBA anti-A (fraction 2)</td>
<td>3/5 1/5 1/5 0/5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>BALB/c anti-C57BL</td>
<td>5/5 4/5 3/5 1/5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>C3H anti-Py 89</td>
<td>5/5 5/5 5/5 5/5</td>
</tr>
</tbody>
</table>

* BALB/c (H-2*) antisera was obtained after 5 weeks intraperitoneal injections of about 10⁶ C57BL (H-2*) spleen and lymph node cells; C3H mouse antisera was obtained after 7 weekly subcutaneous injections of about 10⁶ freshly trypsinized Py 89 (H-2*) tumor cells. BALB/c and C3H antisera had cytotoxic titers of 1/80 and 1/40, respectively. The anti-A (fraction 2) antisera raised in B10.D2 and CBA strain mice are those described in the text and used in the passive enhancement studies.

† 3-month-old CBA male mice and 5-month-old B10.D2 male mice were used as recipients of tumor cells and sera.

Tissue culture-grown Py 89 cells of C57BL origin were inoculated subcutaneously at the time of treatment with various antisera; 0.1 ml. of each antisera, diluted 1/10 was injected intraperitoneally.

Subcutaneous growths of tumor Py 89 in groups 2 and 4 were much larger and had attained a size of >15 mm at the largest diameter, 28 days after grafting.
(b) Passively transferred antisera from recipients of the cell-membrane material or fraction 2, but not from recipients of fraction 3, enhanced the growth of YAA-C1 and Sarcoma I tumors in CBA and B10.D2 mice, respectively; passive immunization was more effective in enhancement of the growth of Sarcoma I.

(c) Enhancement by solubilized H-2\textsuperscript{a} antigens was specific. Passive transfer of antisera from mice immunized with fraction 2 did not enhance tumor Py 89 of C57BL (H-2\textsuperscript{b}) origin; yet, the tumor was readily enhanced by antisera produced by H-2\textsuperscript{b} antigens in suitable recipients (Table 4).

Although these results demonstrate that all important antigenic determinants (H-2\textsuperscript{a}) have been recovered in the soluble material, this does not imply that the transplantation antigen molecules are identical to those present in situ on the membrane of the intact cell. Fragmentation may have occurred, despite the maintenance of immunogenicity of all determinants. Fragmentation may be demonstrated after further purification, characterization, and biologic tests of fraction 2. A critical test is the induction of tolerance.

Nathenson and coworkers (2-4) have shown that prolonged papain digestion of membranes from mouse spleen cells (H-2\textsuperscript{b} and H-2\textsuperscript{a} genotypes) results in fragmentation of H-2 alloantigenic specificities. Although these antigen fragments, partially purified by Sephadex chromatography and polyacrylamide disc electrophoresis, retain their ability to inhibit immune cytolysis of certain alloantigens of limited specificity, they have not been shown to possess true biologic activity. Only a relatively crude papain digest has produced accelerated rejection of allogeneic skin grafts in strain combinations in which differences of loci other than H-2 could not be excluded (16). In later studies (17) by use of aliquots of classes I and II, fragments bearing different series of H-2 specification were demonstrated to have immunogenicity after Sephadex G-150 and carboxymethyl Sephadex purification.

In contrast, the present study demonstrates that when proteolysis by papain is carefully controlled, material from A/J spleen cells, purified by Sephadex G-150 chromatography and confined to a single peak, retains all H-2\textsuperscript{a} alloantigenic specificities, and full serologic and biologic activity. The advantage of limited papain digestion will be confirmed if the methods used here can be successfully applied to the solubilization and purification of H-2 transplantation antigens of other strains of mice.